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TITLE: Descriptive Biomarkers for Assessing Breast Cancer Risk

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**14. ABSTRACT**
The purpose of this research is to determine if exfoliated epithelial cells present in breast milk can be used to assess a woman's individual risk of developing breast cancer and to detect early signs of breast cancer. To accomplish this goal we are collecting breast milk samples from 250 lactating women who either have had a breast biopsy or are scheduled for a breast biopsy. We are isolating the epithelial cells and determining the DNA promoter methylation patterns of several tumor-suppressor genes that are frequently methylated in breast cancer. During the first year we have been extremely successful with subject recruitment and sample collection and processing. We have completed Tasks 1 through 4 and have made significant progress on Tasks 5 and 6. We have not encountered any problems and remain on schedule.

**15. SUBJECT TERMS**
Breast milk; recruitment; promoter hypermethylation; RASSF1

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## Introduction

The purpose of this research is to determine whether the promoter methylation pattern in exfoliated epithelial cells present in breast milk can serve as a reliable means of assessing an individual woman's risk of developing breast cancer. We are collecting breast milk samples from 250 lactating women who either are scheduled for a breast biopsy or have had a breast biopsy in the past. Women provide milk from both the biopsied and non-biopsied breast as well as a copy of their biopsy report. They also complete a health and reproductive history questionnaire. From each milk sample we isolate the epithelial cells from the total cell population and determine the methylation pattern of selected genes using pyrosequencing of bisulfite-modified DNA. Based on published statistics, we expect that roughly 10% or 25 milk samples will come from women with breast cancer. This design provides a unique opportunity to assess the development of cancer-like methylation patterns in premenopausal women who either have breast cancer or a benign lesion and to compare the cells from the diseased breast with those from the healthy breast.

## Body

During the first year of this study the focus has been on subject recruitment and sample collection. We have been extremely successful in subject recruitment. A detailed review of our success in recruitment during the first year is provided in ***Appendix 1*** (a PowerPoint slide show on subject recruitment presented at the second advisory Board meeting on September 21, 2009). The PowerPoint presentation also includes information on the number and type of biopsy reports collected to date. The results shown in ***Appendix 1*** demonstrate that we have ***successfully completed Tasks 1 through 4*** as outlined in the original statement of work.

During the first year we also made ***significant progress on the work described in Tasks 5 and 6***. We processed 102 milk samples, separated the cell populations, isolated DNA, and conducted promoter methylation analyses on bisulfite-modified DNA. Detailed results are in ***Appendix 2*** (a PowerPoint presentation on sample collection and processing presented at the second advisory Board meeting on September 21, 2009). The final slide in this presentation shows that for a single gene, RASSF1, the promoter methylation is significantly greater in DNA from a woman with cancer than in DNA from a woman with benign disease, focal fibroadenoma.

We continue to enter data as it becomes available and keep all data bases up-to-date. We have not conducted any preliminary statistical analyses nor prepared any manuscripts (Task 7).

## Key Research Accomplishments

- Recruited women through three E-Blasts for the Army of Women http://www.armyofwomen.org/
- Contacted over 730 lactation counselors, WIC coordinators, La Leche League coordinators; sent brochures to over 150 mammography centers
- Recruited 165 lactating women who either have had or are scheduled for a breast biopsy
- Completed sample collection (breast milk, questionnaire and biopsy report) for 102 women

- Processed milk samples resulting in ~ 564 cell samples
- Purchased a Pyromark24 and completed training to conduct in-house DNA methylation analyses
- Isolated DNA from 152 cell samples
- Bisulfite-treated 50 DNA samples
- Determined promoter methylation profile RASSF1 in 20 samples
- Created a secure, password-protected WIKI for storing and sharing all data among the study investigators
- Held two Advisory Board Meetings

**Reportable Outcomes**

Received two awards based on the breast milk samples being collected for the present study

1. ***Baystate Health Foundation:*** "Environmental Impacts on Epigenetics: Bisphenol A and Promoter Methylation" (Co-PIs: Douglas Anderton and Kathleen Arcaro)
The goal of this study is to optimize methods for measuring Bisphenol A (BPA) in breast milk and examine the relationship between BPA and DNA promoter methylation in exfoliated breast epithelial cells. IRB protocol allows the use of the milk for other studies.

2. ***Avon Foundation***: "The Presence, Prevalence and Impact of Intracellular *Chlamydia* in Cells from Breast Milk" (PI: Elizabeth Stuart; Co-PI: Kathleen Arcaro)
The goal of this study is to determine the relationships among infection with *Chlamydia*, promoter methylation and breast cancer risk. We will examine *Chlamydia* infection in the breast milk we collect from the participants in the present study. IRB protocol allows the use of the milk for other studies.

Submitted several proposals based on the preliminary results of the present study. These proposals were not funded but the reviews were helpful in preparing the proposals for resubmission.

1. ***Susan G. Komen Foundation***: "Promoter Methylation in Breast Milk from African American Women" (PI: Kathleen Arcaro)
The goal of this study was to expand our current study to include African American Women.

2. ***NIH Challenge Proposal***: "Biomarkers for Assessing Increased Risk and Early Detection of Breast Cancer in African American women" (Co-PIs: Kathleen Arcaro & Douglas Anderton)
The goal of this study was to expand our current study to include African American Women and to examine additional biomarkers of breast cancer risk.

**Conclusion**

During the first year of this award we quickly established the tools and protocols needed for successful recruitment and sample collection. We are well on our way towards recruiting the 250 women within the time frame originally outlined. Our preliminary results are extremely

exciting in that we detected a methylation pattern consistent with breast cancer in DNA from the epithelial cells in the breast milk of a woman with breast cancer.

Recently the United States Preventative Task Force issued new guidelines for screening mammography. These new guidelines specify that for women at average risk, mammographic screening for breast cancer should begin at age 50 instead of the previously recommended age 40. Earlier mammographic screening continues to be recommended for women at high risk. In this context, it is particularly important to be able to identify women at high risk who may benefit by early initiation of mammographic screening. We think we will be able to use breast milk to identify lactating women at increased risk of breast cancer.

**References**

None

**Appendices**

1. PowerPoint slides on "Recruitment" presented at the second Advisory Board meeting on September 21, 2009

2. PowerPoint slides on "Sample Collection and Processing" presented at the second Advisory Board meeting on September 21, 2009

# Breast Cancer Risk Assessment

Advisory Board Meeting
September 21, 2009

Recruitment

## Recruitment Strategy – Web-based

- AOW – Two e-blasts have produced 233 subject contacts
- Listserves – 4
- Message boards - 9
- Blogs >20
- Paid advertising 1 (Facebook)
- Other web-sites

## Recruitment Strategy - Conventional

- Lactation counselors, WIC coordinators, LLL coordinators - have sent emails to 732
- Breast Imaging Centers - have mailed brochures to 157
- OB/GYN offices – have contacted 27
- Professional Organizations - have contacted 6 (Avon, ACS, ACOG, SBS, LLL, YSC)

## Contact and Recruitment Numbers

| | |
|---|---|
| Number of Women Contacted | 293 |
| Number of Women Who Responded | 198 |
| Number of Women Recruited | 165 |
| Number of Women Completed | 102 |

## Categories of Women Recruited

| Category | Number of Women (number sent boxes) | Number of Milk Samples Returned | Number of Biopsy Reports Received |
|---|---|---|---|
| Biopsy Planned | 8 (8) | 6 | 3 |
| Previous Biopsy | 148 (143) | 125 | 90 |
| No Biopsy | 10 (10) | 10 | na |
| **Total** | **199** | **141** | **93** |

## Breast Cancer Risk Assessment

Biopsy Results

## Biopsy Types

- FNA = 12
- Core Biopsy = 27
- Surgical Biopsy = 53
- Unknown = 1

## Malignant Diagnoses

- Ductal carcinoma in situ (DCIS) = 1
- Invasive ductal carcinoma (IDC) = 2
- DCIS + IDC = 1
- IDC+ invasive lobular carcinoma = 1

- We also have received frozen milk samples from 3 additional women who have been diagnosed with breast cancer

# Biopsy Diagnoses

American Cancer Society classifies benign diagnoses into three levels of risk

- Non-proliferative lesions – none or very small elevation of risk (fibrocystic disease (fibrosis and/or cysts) mild hyperplasia (an abnormal overgrowth of cells) , adenosis (non-sclerosing, or non-hardening of tissue) , simple fibroadenoma , phyllodes tumor (benign) , a single papilloma , fat necrosis , mastitis , duct ectasia , other benign tumors (lipoma, hamartoma, hemangioma, neurofibroma) : N=75
- Proliferative lesions without atypia – 1/1/2 – 2 x elevation of risk (usual ductal hyperplasia (without atypia) complex fibroadenoma . sclerosing adenosis , several papillomas or papillomatosis . radial scar : N=13
- Proliferative lesions with atypia – 3-5 x elevation of risk (atypical ductal hyperplasia , atypical lobular hyperplasia ) *: N= 1

* We also include lobular carcinoma in situ (LCIS) in this highest risk category

# Biopsy Diagnoses Non-Proliferative Lesions

| Category | N | Category | N |
|---|---|---|---|
| adipose tissue | 1 | fibrosis | 10 |
| angiolipoma | 1 | harmartoma | 1 |
| benign | 2 | inflammatory cycst | 1 |
| cyst | 3 | lymph node | 2 |
| duct ectasia | 1 | lactational changes | 9 |
| fat necrosis | 1 | lipoma | 1 |
| FCC | 6 | mastitis | 2 |
| fibroadenoama | 26 | negative | 3 |
| fibroadenosis | 1 | phyllodes tumor | 1 |

# Biopsy Diagnoses Proliferative Lesions with and without Atypia

**Without Atypia**

| Category | N |
|---|---|
| complex fibroadenoma | 1 |
| ductal hyperplasia | 8 |
| FCC proliferative | 1 |
| radial scar | 1 |
| sclerosing adenosis | 2 |

**With Atypia**

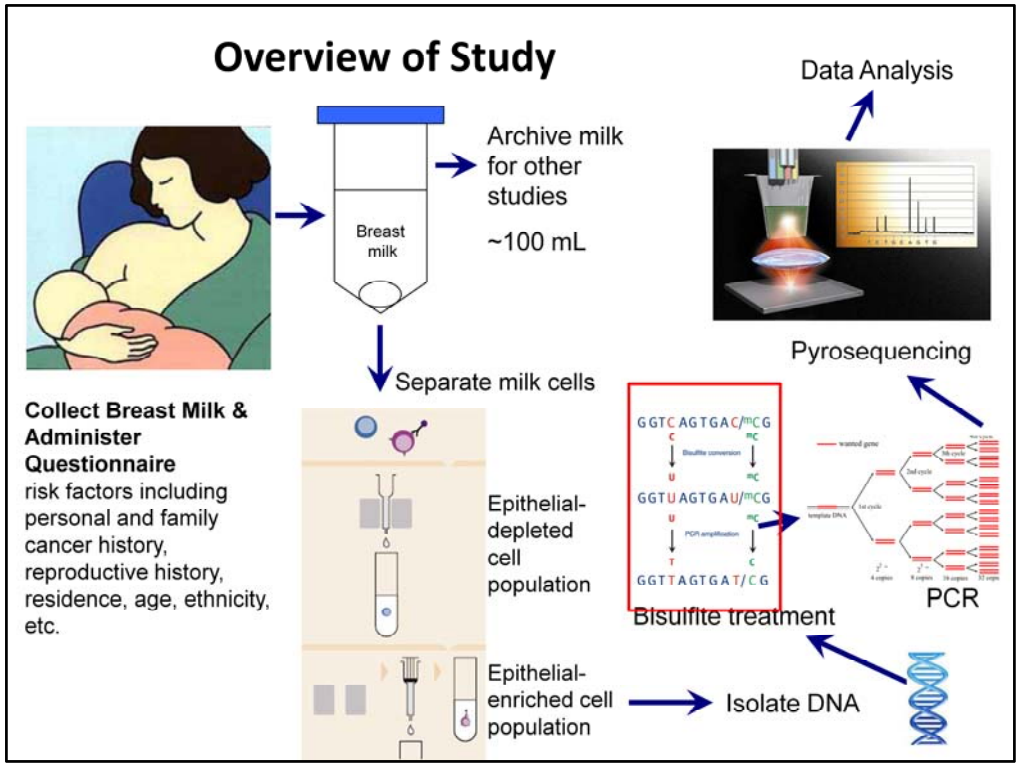
| Category | N |
|---|---|
| atypical ductal hyperplasia | 1 |

# Molecular Biomarkers for Assessing Breast Cancer Risk

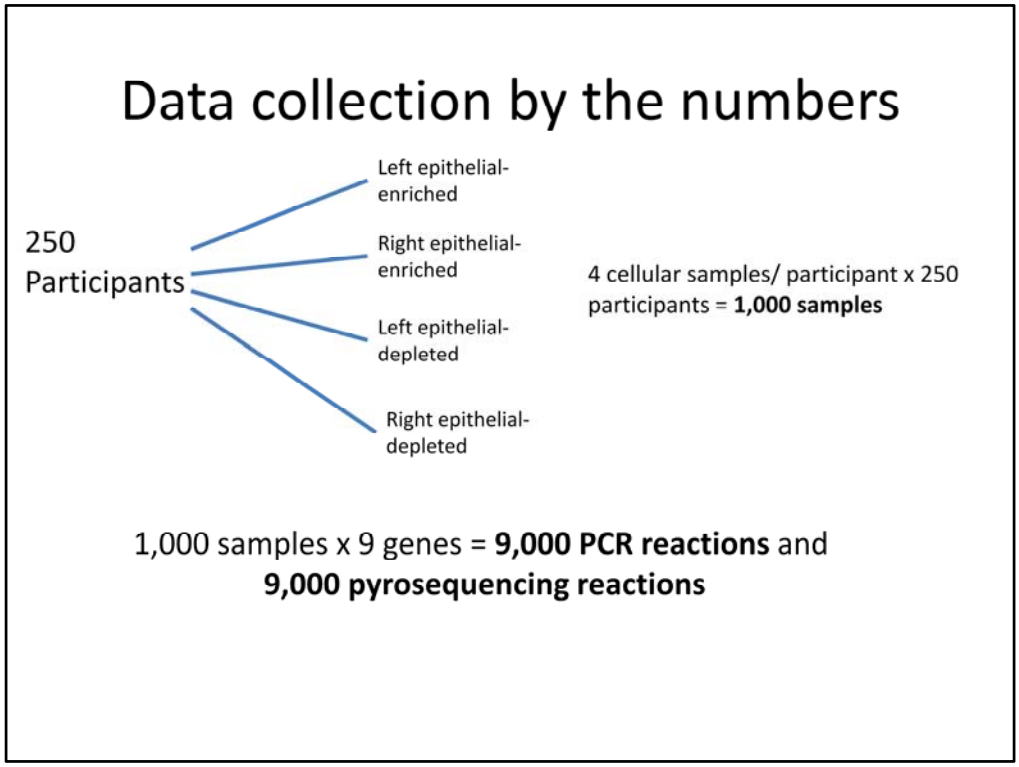
Second Advisory Board Meeting

September 21, 2009

Sample Collection and Processing




1. Sarah contacts potential participants, sends us addresses of women who qualify for the study.
2. Breast milk collection kit mailed to participant.
3. Kit returned to Amherst with samples.
4. Milk processed, epithelial enriched and depleted cell fractions obtained.
5. Genomic DNA extracted from cell fractions.
6. Genomic DNA converted to bisulfite treated DNA. BS treatment results in a sequence change so that we may detect methylation. KEY STEP: BS treatment has been optmized for these samples.
7. Amplify bisulfite treated DNA for specific gene of interest.
8. Pyrosequence amplified DNA.




Ideally we will get 250 participants for this study, we currently have about 140 milk samples. If we are able to obtain all 250 and each woman gives us a sample from both her left and right breast this will result in 4 cellular fractions for each participant. 1,000 total samples.

Each of these samples then must be amplified for each of the 9 genes of interest and then pyrosequenced to determine levels of methylation for each gene. This will be 9,000 PCR and pyrosequencing reactions.

This will give us very exciting data and we are already well on our way sample processing…. (next slide)

## Data Collection Progress

Since September 30, 2008

| Processing Step | Number Completed |
|---|---|
| Cellular isolation | 141 participants resulting in approximately 564 cell samples |
| DNA isolation | 152 |
| Bisulfite treatment | 50 (12 participants) |
| RASSF1A pyrosequencing | 20 (5 participants) |

We have 141 participants that results in a little less than 564 samples, (Some women may not have been able to donate from both the right and left breast, resulting in slightly less than 562 cellular samples).
Of these samples Beth Punska has already isolated the DNA from 152 (about a quarter). She has only been isolating DNA since August 4, 2009.

We have completed BS treatment on about a third of the isolated DNA samples.

The first gene that we are going to examine is a tumor suppressor gene RASSF1A which has been shown to be methylated in breast cancer and other cancers. We have completed pyrosequencing for about 20 samples.

Very pleased with how much we have accomplished in the processing of these milk samples and I am confident that we will be able to complete this project according to our schedule.

I would like to focus a little more on the first step in this process, isolating the cells from these fresh breast milk samples…

# Cell Counts

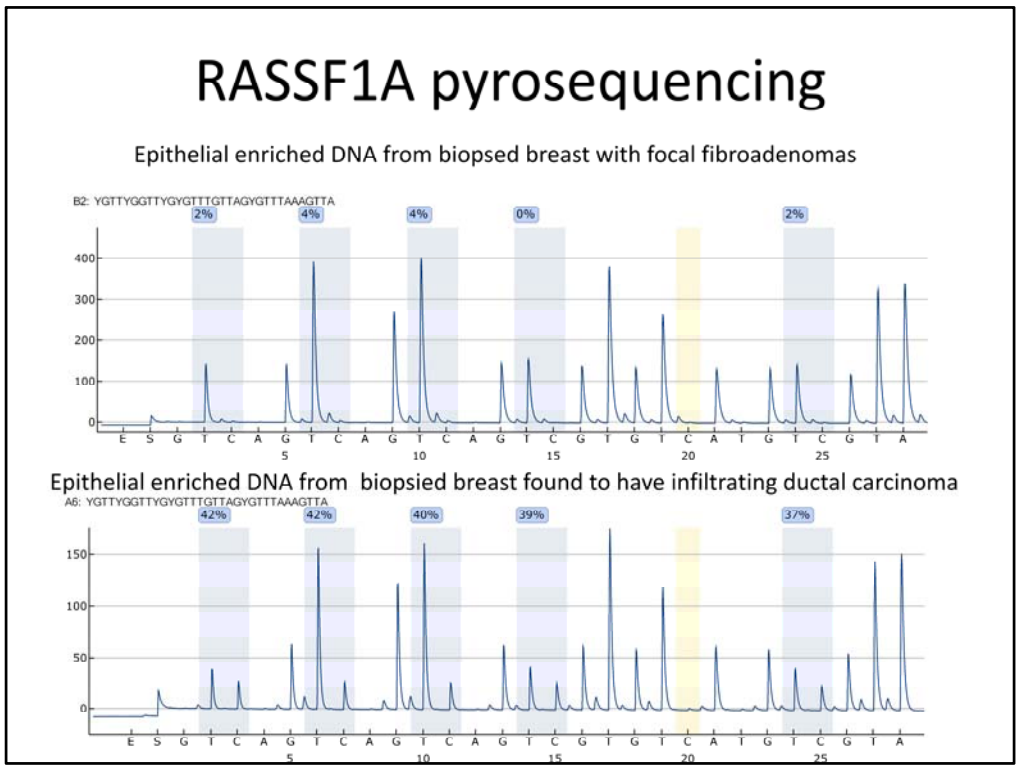
| N=141 | Average | Median | Low | High |
|---|---|---|---|---|
| Left Epithelial Enriched | 893,000 | 385,000 | 0 | 6,000,000 |
| Left Epithelial Depleted | 1,100,000 | 280,000 | 0 | 10,000,000 |
| Right Epithelial Enriched | 973,000 | 330,000 | 0 | 5,000,000 |
| Right Epithelial Depleted | 1,200,000 | 390,000 | 0 | 17,000,000 |

Cell counts vary a lot occassionally very small samples (10 ml) can provide lots of cells. Usually the more milk we get the larger total cell pellet we will get, however the percentage of the total cell population that is epithelial varies a lot.

You can see that on average we tend to get less cells in the enriched fraction although this is probably due to some outliers in the depleted fractions that had over 10,000,000 cells.

We save the cell samples that give us a zero count and have been able to isolate DNA from some of those samples, indicating that although there are very few cells when we count an aliquot, there may still be some recoverable DNA.
We are encouraged that we can get this many cells from our breast milk samples.

Moving on to some preliminary results from pyrosequencing…




The first pyrogram is from a woman who is at low risk, she had a biopsy which revealed focal fibroadenomas. You can see by these blue highlighted boxes that she has very low DNA methylation at these CpG sites.

In contrast, this second pyrogram is from a woman who had a biopsy after giving this sample and was diagnosed with infiltrating ductal carcinoma. You can see here that her levels are much higher at 40% methylation.